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Immunology

Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning

(antibody repertoires/immunotherapy/tetanus toxoid)

MATS A. A. PERSSON*†, ROGER H. CAOTHIEN*, AND DENNIS R. BURTON*‡§

*Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037; †Department of Medicine, Karolinska Institute, Karolinska Hospital, Box 60500, S-104 01 Stockholm, Sweden; and ‡Krebs Institute, Department of Molecular Biology and Biotechnology, The University, Sheffield S10 2TN, United Kingdom

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ABSTRACT **Combinatorial libraries of antibody heavy and light chains derived from the peripheral blood lymphocytes of an individual immunized with tetanus toxoid have been expressed in *Escherichia coli* by using phage λ vectors. Screening of the libraries allowed identification of a large number of human monoclonal Fab fragments specific for tetanus toxoid. Initial studies suggested considerable sequence diversity in these antibodies. The method should allow the generation of many human monoclonal antibodies of interest and the dissection of human humoral immune responses.**

Mouse monoclonal antibodies are readily generated by the fusion procedure of Köhler and Milstein (1). However, for therapeutic applications, human monoclonal antibodies are preferred. Despite extensive efforts, including production of heterohybridomas (2), Epstein–Barr virus immortalization of human B cells (3) and "humanization" of mouse antibodies (4), no general method comparable to the Köhler–Milstein approach has emerged for the generation of human monoclonal antibodies (reviewed in ref. 2). Previously, a procedure was described for the production of mouse monoclonal antibodies based on antigen selection from a combinatorial library of the mouse antibody repertoire cloned in phage λ (5). The procedure has recently been successfully applied to the isolation of mouse monoclonal antibodies specific for influenza virus hemagglutinin (6). The extension to human antibodies is not trivial. Mice can be hyperimmunized with antigen and the spleen, a rich source of antibody-producing cells, can be removed. Humans can be immunized or are immune to many antigens but only peripheral blood, a poor source of antibody-producing cells, is readily available.

Here we show that the repertoire cloning approach can be applied for the generation of human monoclonal antibodies from peripheral blood lymphocytes (PBLs) and we speculate that, given appropriate donor selection, this may effectively constitute a general route to such antibodies.

MATERIALS AND METHODS

Immunization and Lymphocyte RNA Preparation. A healthy volunteer who had received the most recent tetanus toxoid immunization 18 months before this experiment was given a booster injection of 0.5 mg of diphtheria/tetanus toxoid intramuscularly. Seven days later, he was subjected to leukapheresis, processing 5 liters of blood resulting in 2.1×10^9 lymphocytes after additional density-gradient separation (Ficoll-Paque; Pharmacia); 0.5×10^9 cells each were taken for three parallel experiments: (*i*) untreated PBLs, RNA was isolated immediately by a guanidinium isothiocyanate method (7); (*ii*) *in vitro* antigen stimulation, in which the cells were cultured in the presence of tetanus toxoid (10 μg/ml) for 3 days in RPMI 1640 medium with 10% human AB serum (GIBCO), after which the RNA was isolated as described above; (*iii*) panned antigen reactive cells, lymphocytes were incubated for 45 min with 60 nM biotin-tetanus toxoid, washed twice, and then poured onto Petri dishes coated with streptavidin and blocked with bovine serum albumin (8), incubated for another hour at +4°C, and then washed extensively. After the last wash, the plates were shaken dry and 2 ml of 3 M guanidinium isothiocyanate with 2-mercaptoethanol was added as the first step of RNA isolation according to the procedure mentioned above. After completion of the isolation, RNA was stored precipitated by 50% isopropanol in 3 M guanidinium isothiocyanate with 2-mercaptoethanol at −20°C until used for reverse transcription.

Library Construction. Total RNA (20–50 μg) was added to 60 pmol of either γ1 or κ-chain 3′ primers (Table 1) and heated at 70°C for 10 min. The mixture was then used in a 50-μl reverse transcription reaction (buffer; BRL) containing 200 μM each dATP, dCTP, dGTP, and dTTP and 600 units of reverse transcriptase (SuperScript; BRL), which was incubated at 37°C for 1 hr. The RNA·cDNA mixture (3 μl) was then used in 100-μl PCR reaction mixtures (buffer containing 10 mM $MgCl_2$; Promega) containing all four dNTPs at 60 μM, 5 units of *Taq* polymerase (Promega), and 60 pmol of the appropriate 5′ and 3′ primers (Table 1). The reaction mixtures were then subjected to 35 rounds of amplification (Perkin–Elmer/Cetus thermal cycler) at 91°C for 1 min, 52°C for 2 min, and 72°C for 1.5 min followed by a final incubation at 72°C for 10 min. An aliquot of the reaction mixture was run on a 2% agarose gel. Providing the gel indicated successful amplification, the remainder of the RNA·cDNA was amplified, the PCR products of a common 3′ primer were pooled, phenol/chloroform extracted, and ethanol precipitated. The resulting DNA was digested with the appropriate pair of restriction enzymes at 5-fold excess, extracted, and precipitated again before running on a 2% agarose gel. The desired band (≈650 base pairs) was purified with GeneClean (Bio 101) and DNA was ligated into the vector arms λHC2 or λLC1 (5) or λLC2 (see text) in roughly equimolar amounts. An aliquot of the ligation mixture was packaged with Gigapack II Gold (Stratagene) and the resulting library was titered and assayed for the expression of heavy chain (anti-decapeptide; ref. 5) or light chain (anti-κ; Southern Biotechnology Associates, Birmingham, AL). These libraries were amplified and phage DNA was prepared from them by using a glycerol step-gradient method. About 10–15 μg of this DNA was digested with the appropriate enzyme, treated with phosphatase, digested with *Eco*RI, religated, and packaged as de-

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Abbreviations: V_H domain, variable heavy-chain domain; C_κ, constant region κ chain; CDR, complementarity determining region; FR, framework region; PBL, peripheral blood lymphocyte.

§To whom reprint requests should be addressed.

Table 1. Primers used for the amplification of human γ1 (Fd) and κ sequences

γ1	
VH1a	5′-CAGGTGCAGCTCGAGCAGTCTGGG-3′
VH3a	5′-GAGGTGCAGCTCGAGGAGTCTGGG-3′
VH1f	5′-CAGGTGCAGCTGCTCGAGTCTGGG-3′
VH3f	5′-GAGGTGCAGCTGCTCGAGTCTGGG-3′
CG1z	5′-GCATGTACTAGTTTTGTCACAAGATTTGGG-3′
CONGa	5′-TCCACCAAGGGCCCATCG-3′
κ	
VK1a	5′-GACATCGAGCTCACCCAGTCTCCA-3′
VK3a	5′-GAAATTGAGCTCACGCAGTCTCCA-3′
CK1a	5′-GCGCCGTCTAGAACTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCAAG-3′
CK1z	5′-GCGCCGTCTAGAACTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCGATCTCAG-3′
CONK	5′-ACTGTGGCTGCACCATCTG-3′

VH1a and VH3a are 5′ primers for amplification of the heavy chain designed to maximize homology with the V_H1 and V_H3 subgroup families (9), respectively, although considerable cross-priming of other subgroups is expected. The *Xho* I site for cloning into the heavy-chain vector λHC2 is shown underlined. VH1f and VH3f are analogous primers that give a slightly different N terminus to the corresponding heavy-chain protein. CG1z is the 3′ primer for the γ1 chain and corresponds to part of the hinge region. The *Spe* I site for cloning into λHC2 is underlined. CONGa is a control 5′ primer and corresponds to the 5′ end of the $C_\gamma1$ domain. Amplification with this primer gives a band at 300 base pairs corresponding to the $C_\gamma1$ domain. VK1a and VK3a are 5′ primers for the κ chain analogous to the heavy-chain primers described above, with the *Sac* I site for cloning into the light-chain vectors λLC1 or λLC2 shown underlined. CK1a is a 3′ primer corresponding to the 3′ end of the light chain, *Xba* I site underlined. The base substitution G → A, which removes the *Sac* I site occurring normally in the $C_\kappa1$ gene, is shown in boldface. This results in the replacement of Ser-203 by Leu in the cloned protein. CK1z is a primer with the substitution G → T (boldface), which leaves the amino acid sequence unchanged. CK1z is not as effective in amplification as CK1a under the conditions in Fig. 1. CONK is a control 5′ primer and corresponds to the 5′ end of the $C_\kappa1$ domain.

scribed (5). The resulting combinatorial library was examined for coexpression of heavy and light chains before amplification and storage at 4°C in 0.3% chloroform.

Library Screening for Antigen Binding. Tetanus toxoid was labeled by using activated alkaline phosphatase as described by the manufacturer (Pierce). Typically XL1-blue cells were infected with 20,000–30,000 phage from a combinatorial library and plated out; duplicate filter lifts were collected over successive 8- to 12-hr periods at ambient temperature. The filters were cleaned in 0.5% Tween 20/phosphate-buffered saline (PBS), blocked in 1% bovine serum albumin/PBS, labeled for 3–4 hr in 1 nM tetanus toxoid/alkaline phosphatase, washed three times in 0.5% Tween 20/PBS for 5 min each time, and, finally, washed in PBS before color development (NBT and BCIP; BRL) in 0.1 M Tris·HCl, pH 9.5/0.05 M NaCl/0.1 M $MgCl_2$. Duplicating positives were cored, replated, and rescreened. Generally, a third round of screening was required to permit the isolation of a pure phage.

Nucleic Acid Sequencing. Plasmids containing heavy- and light-chain cDNA were excised from purified phage with helper phage R408 (Stratagene). Plasmids were propagated in XL1-blue cells in ampicillin-containing medium and double-stranded DNA was purified on a CsCl gradient. Sequencing was by the dideoxynucleotide method with T7 DNA polymerase (10).

Inhibition ELISAs. Phagemids were excised from positive clones, used to transform XL1-blue cells, grown until the OD reached 1, and treated overnight at room temperature with 2 mM isopropyl β-D-thiogalactopyranoside (5). Pelleted cells were sonicated and supernates were titered in a checkerboard assay to determine optimal conditions for an inhibition ELISA (11). For this assay, the supernates were incubated in tetanus toxoid-coated wells (coating concentration, 10 μg/ml) along with serially diluted free tetanus toxoid overnight to allow equilibration. The plate was stringently washed with PBS/0.05% Tween 20 four or five times. Secondary antibody goat anti-human IgG $F(ab')_2$ labeled with alkaline phosphatase (Pierce) was added and incubated for 1 hr at 37°C. The plate was washed again with PBS and developed with *p*-nitrophenyl phosphate.

RESULTS

Library Construction. One of us was given a tetanus toxoid booster injection and leukapheresed 7 days later; mononuclear cells were isolated by density-gradient centrifugation. The leukapheresis was used to provide a maximum number of cells to allow diverse investigations and would not be necessary for the construction of the libraries as described here. The cells were divided into three parts. One was used directly for the preparation of total RNA (untreated cells), a second was cultured for 3 days in the presence of antigen (stimulated cells), and a third was panned against antigen (panned cells) before RNA isolation.

γ1 (Fd part) and κ immunoglobulin chains were PCR-amplified after reverse transcription from this RNA. Four heavy-chain 5′ primers were used in the amplification. Two were designed to maximize homology with the variable domain heavy-chain $(V_H)1$ subgroup and two with the V_H3 subgroup families (9). However, considerable cross-priming and priming of other subgroups is expected. Two primers for each subgroup (Table 1, -a and -f) were used corresponding to heavy-chain proteins of slightly different N terminus. This was done because the design of the vector λHC2 is such that some change to the human heavy chain at the N terminus is inevitable. By having two possibilities we hoped to decrease the chance of artifact. In fact, both N termini can support antigen binding (see below). The smaller number of primers used compared with mouse (5) reflects the lower variability seen in human 5′ heavy-chain sequences. Two κ-chain 5′ primers were used and one of two 3′ primers was used, which were exceptionally long to eliminate a naturally occurring *Sac* I site in the human constant region κ chain $(C_\kappa)1$ domain exon. [*Sac* I is one of the enzymes used for cloning inserts into the light-chain vector λLC1 (5).] Typical PCR amplification of heavy and light chains is shown in Fig. 1.

PCR-amplified products were digested with the appropriate pair of enzymes and used to construct heavy- and light-chain libraries in the phage λ vectors λHC2 and λLC1 (5) or λLC2, respectively. (λLC2 is identical to λLC1 except that a defect in the *Not* I site in the latter has been corrected.) Isolated phage DNA from these libraries was cut and "crossed" to give combinatorial libraries of 1×10^5 members (untreated cells), 6×10^5 members (stimulated cells), and 0.5

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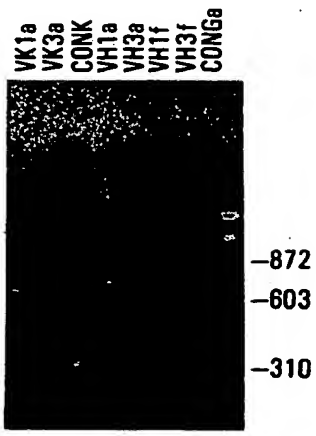

FIG. 1. PCR amplification of γ1 and κ chains from human lymphocyte RNA. Primers are described in Table 1. Amplification conditions are described in *Materials and Methods*. The amplification shown is from cDNA originating from stimulated cells.

$\times 10^5$ members (panned cells) coexpressing heavy and light chains. In such libraries, phage were typically 55–85% heavy-chain positive (as measured by expression of decapeptide tag) and 70–80% light-chain positive.

Identification of Antigen Binding Clones. The Fab libraries were screened for antigen binding by using tetanus toxoid conjugated with alkaline phosphatase. Positive clones were counted as those that duplicated on a primary screen and gave multiple positives in a secondary screen as shown in Fig. 2. The frequency of positives was 1 in 6000 for untreated (17 antigen-binding clones), 1 in 5000 for stimulated (120 clones), and 1 in 4000 for panned (13 clones) cell libraries.

The specificity of antigen binding was suggested by filter-lift competition experiments with unlabeled antigen with apparent inhibition constants in the range of 10^{-7}–10^{-9} M. Briefly, filter lifts from 10 positive plaques were exposed to labeled tetanus toxoid in the presence of increasing concentrations of unlabeled toxoid essentially as described (5) when half-maximal signal intensity was observed in the concentration range of 10^{-7}–10^{-9} M toxoid. The specificity was confirmed by inhibition ELISA studies (11) of the binding of antigen to Fab fragments (Fig. 3). Excised plasmids from five positive clones were used to transform *E. coli* and bacterial supernates containing Fab fragments were concentrated. ELISA wells were then coated with tetanus toxoid, a fixed concentration of Fab was added, and the concentration of free added toxoid was varied. After equilibration and washing, the bound Fab was determined from a labeled anti-$F(ab')_2$. For one of the clones, Fab was purified from the bacterial supernate on an anti-$F(ab')_2$ column and antigen

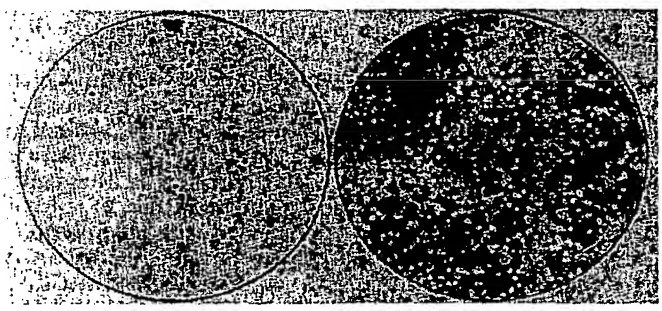

FIG. 2. Library screening for antigen binding. Identification of positive plaques with labeled antigen in a colorimetric assay. A typical duplicate secondary screen is shown where positive plaques are clearly visible against a background of negative plaques.

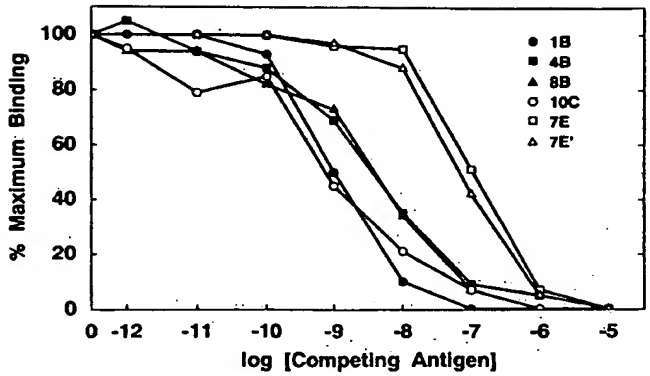

FIG. 3. Specificity of antigen binding shown by competitive ELISA. Experiments were carried out with bacterial supernates except for 7E', in which a purified Fab preparation was used.

binding was examined in an inhibition ELISA. The results were very similar to those obtained when the corresponding supernate was used. Bacterial supernates containing negative clones as judged by the phage screening assay showed no reactivity with tetanus toxoid in ELISA experiments.

These studies indicated apparent inhibition constants in the range 10^{-7}–10^{-9} M and imply monomer Fab–antigen binding constants of the order of 10^7–10^9 M^{-1}. These are consistent with a specific interaction and indicate that the molecules we have identified are not polyspecific antibodies (3), which generally bind antigen at affinities of 10^6 M^{-1} and less. Finally, we screened a combinatorial library of 10^6 antibodies from an individual not injected with tetanus toxoid booster and did not identify a single positive, again supporting the notion that the antibodies identified are specific.

Analysis of an Antigen-Binding Protein. The putative Fab described above, which was isolated on an anti-$F(ab')_2$ column and which bound to tetanus toxoid (Fig. 3), was run on a nonreducing SDS/polyacrylamide gel. It gave a single band of ≈50 kDa. On an immunoblot from a reducing gel anti-decapeptide antibody developed a band of ≈25 kDa and anti-κ developed a band of ≈22 kDa (data not shown). The results indicate the binding protein to have the properties consistent with an Fab fragment.

Sequencing of Antigen-Binding Clones. To look at the diversity of antigen-binding clones, nucleic acid sequences of a limited number of clones isolated from the stimulated cell library were determined. Four κ chains were examined and all belonged to the VκIII subgroup (9) but were clonally distinct in that the complementarity-determining region 3 (CDR3) showed multiple nucleic acid sequence differences. Comparing the framework 3 (FR3) region, two sequences were identical, whereas the others showed between 5 and 10 amino acid differences between pairs. Four complete heavy chains were examined and all clearly arose from different germ-line genes, three belonging to the V_HIII and one to the V_HI subgroup. Two sequences corresponded to the a-set of primers and two corresponded to the f-set (Table 1). Eight heavy-chain CDR3 regions were sequenced and only two pairs appeared clonally related—i.e., there were six distinct heavy chains. The length of CDR3 varied between 6 and 22 amino acid residues (Fig. 4). This shows that the method allows the selection of diverse specific antibodies and should permit a detailed analysis of the human humoral response. A further point of interest is that, as observed by Caton and Koprowski (6), we identified a binding clone with an identical heavy chain but two different light chains.

DISCUSSION

We have shown that human monoclonal Fab fragments binding tetanus toxoid with high affinity can be generated

Clone	FR3	CDR3	FR4
1A	YYCAR	DHEDSLGGIWGYLEY	
1B	YYCAR	DHEDSLGGIWGYLEY	WPQG
4A	YYCAR	APYDFWNGYYLDY	WGQG
4B	YYCTT	GVTLDY	WGQG
5B	YYCAK	ASRQCVAEYYFDFDY	WPQG
6A	YYCAK	AARQWLAEYYFDY	WRQG
7D	YYCAR	HGSQREITVFGTSDFFPYAMDI	WGQG
8C	YYCTT	GITLDY	WGQG

FIG. 4. Heavy-chain CDR3 amino acid sequences from antigen-binding clones. Parts of the flanking framework regions FR3 and FR4 are also shown. Only clones 1A and 1B (identical) and 4B and 8C (1 base difference) showed significant homology when nucleic acid sequences were compared.

from a combinatorial phage library of heavy and light chains prepared from the PBLs of an immunized donor. The titer of specific antibody in the serum of the patient at the time of cell harvesting was 1:14,000. If this titer following a booster injection can be taken as a rough indicator of frequency of antigen binders to be expected in the corresponding library, then human antibodies of great interest against a wide range of infectious agents should now be accessible via repertoire cloning. It may be that a booster injection will be necessary to ensure the presence of specific B cells at an acceptable frequency in the peripheral blood. At later times, the antibody titer may still be high but the specific B cells localized in sites such as lymph nodes and spleen and no longer at sufficient concentration in peripheral blood.

Interesting comparisons can be made with the other repertoire cloning studies carried out on mice immunized with the hapten NPN (5) or influenza hemagglutinin (6). The diversity of the response we observe is greater than that described for hemagglutinin, which was very restricted (6), or that observed for NPN (A. S. Kang, personal communication). This could reflect differences in antigen, species, immunization protocol, or RNA tissue source. More studies will be needed to decide.

The frequency of binders observed for the three repertoire cloning investigations is fairly similar. Given the greater diversity observed in this study compared with the hemagglutinin study this implies a higher incidence of heavy and/or light chains originating from antigen-specific clones ("functional chains") in the tetanus case. The reasoning is as follows. Caton and Koprowski (6) observed, by hybridization studies, essentially a single functional heavy chain at a frequency of ≈ 1 in 50 and a light chain at a frequency of ≈ 1 in 275. Therefore, the random combination of heavy and light chains in the phage library should generate specific functional antibodies at a frequency of ≈ 1 in 14,000, which is reasonably close to the observed value of 1 in 12,500. The initial sequence data on the tetanus toxoid binding clones indicates the presence of a number of different functional heavy and light chains. It is probable that only a limited number of combinations of these chains will give functional antibodies. If, therefore, the frequency of tetanus toxoid functional chains was the same as that described for hemagglutinin then the frequency of functional combinations—i.e., tetanus toxoid binders—would be less. In fact, because of the multiplicative factor involved in the combinatorial approach, the frequency of binders would be orders of magnitude less. The similarity in frequency therefore implies the tetanus toxoid library is richer in functional heavy- and light-chain sequences. Again, a number of factors could be important here, although one could hypothesize that PBLs following a booster injection may be a more biased (and therefore more favored for repertoire cloning) source of antigen-specific IgG RNA than spleen.

A number of other antibodies against self antigens useful in immunosuppressive therapies may be accessible via repertoire cloning from the lymphocytes of patients with various autoimmune disorders. For antibodies of low titers, enrichment of source lymphocytes secreting specific antibody may be desirable to increase the frequency of binders in the library. We achieved only moderate success by either antigen stimulation or antigen panning. In the former case, the stimulation protocol may not have been optimal and one would like further studies to make any definitive conclusions. In the latter case, an important factor may be that cells expressing high levels of surface antibody tend to be relatively poor secretors of that antibody. An alternative strategy for low titer antibodies is to greatly enhance the number of clones that can be screened by the expression of antibody on the surface of bacteria or phage allowing affinity selection of positive clones. In any case, both of these areas are worthy of study to extend the scope of repertoire cloning. Surface expression might also stimulate attempts to generate high-affinity antibodies by rounds of mutation and selection from the low-affinity antibodies arising from *in vitro* immunization.

Finally, tetanus may be more than a model system. In areas where vaccination with tetanus toxoid is not generally available fatal cases due to *Clostridium tetani* are not uncommon, in particular among the newborn. Administration of large doses of antibody originating from human serum is the regimen of choice (12). The specific human antibodies or antibody fragments described here may provide a more satisfactory therapy.

Note. During the later stages of preparation of this manuscript, a report appeared describing the generation of human monoclonal antibodies against tetanus toxoid by the combinatorial approach (13). This report used a κ-chain PCR primer that did not eliminate the *Sac* I site in human κ chain described above. Therefore, the integrity of the light chains and antibodies described in that study must be open to question.

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